H1650 cells: 1) anti-EpCAM-PE, anti-CD45-FITC, and 4',6-diamidino-2-phenylindole (DAPI), and 2) anti-cytokeratin-PE, anti-CD45-FTIC, and DAPI. Cytokeratins (CKs) are proteins found in the cytoskeletons of epithelial cells. There are both basic (CK1-9) and acidic (CK10-20) types of cytokeratin, and they are generally found in pairs. CK expression can vary across different organs or tissues, so generally a CK staining kit contains a mixture of anti-CKs specific to a few CK types. In these experiments, a mixture of anti-CK-14, 15, 16, and 19 was used. CK-19 is expressed by many carcinomas. Which varieties of anti-CK used, however, will eventually need to be optimized to result in efficient staining of CTCs regardless of origin. Anti-CK dyes require membrane permeabilization, which can be accomplished by incubating the cells in a detergent, such as 0.2% Triton surfactant. This results in a loss of cell viability. Thus, anti-EpCAM-PE was evaluated as a potential surface stain for CTCs.

[0060] DAPI is a nuclear stain that binds strongly to DNA. DAPI can pass through an intact membrane of a living cell, but passes more efficiently after cell fixation. When bound to DNA, the fluorescence of DAPI is increased about 20-fold, with excitation/emission maxima at 358/461 nm.

[0061] Merged images of an H1650 cell and a WBC on a glass slide stained with anti-EpCAM-PE, anti-CD45-FITC, and DAPI were inspected. As expected, the H1650 cell fluoresces red and blue, while the WBC fluoresces green and blue. RBCs exhibit weak background fluorescence, as they do not express EpCAM or CD45, nor possess nuclei.

[0062] To evaluate the 3-color staining of tumor cells, bare H1650 were spiked into 0.5 mL of whole blood at a concentration of ~1000 H1650/mL. Magnetic labeling was performed using Protocol 1 discussed above, and cell suspensions were separated at 5 ml/hr using a 50 μm square pore sifter. Following separation and washing with 0.5 mL PBS buffer, staining was achieved by first flowing through the sifter a 0.5 mL solution of 0.2% Triton x-100 surfactant in PBS at 5 ml/hr to permeabilize the cell membranes. 0.5 mL of PBS buffer containing 50 μL of anti-CD45-FITC stock was then flowed through the sifter at 3 ml/hr, followed by a wash with 0.5 mL PBS buffer at 5 ml/hr. 0.5 mL of PBS buffer containing 50 μL of anti-EpCAM-PE or anti-CK-PE stock was flowed through the sifter, followed by a wash with PBS. 0.5 mL of PBS containing 10 μg/mL DAPI was flowed through the sifter, followed by wash with PBS. Sifters were then removed from their holders and placed under the optical microscope for fluorescent imaging.

[0063] It was found in order to visualize the cells on the sifter surface, a high magnification objective (50×) was needed. Previous enumeration experiments with H1650 labeled with Green CellTracker™ dyes utilized a 10× objective, which allowed rapid, manual scanning over the sifter surface. For weaker surface antibody conjugated dyes, a higher magnification lens with a higher numerical aperture resulted in brighter images of cells. Only one pore array can be viewed at one time, and exposure times of 1-5 seconds are required to attain adequate brightness.

[0064] In a pore array following capture of bare H1650 spiked in whole blood, magnetically labeled with Protocol 1, and fluorescently labeled as mentioned above, fluorescence from DAPI is sufficiently strong to view through the microscope eye-piece. Fluorescence from FITC and PE require 1 second exposure times with the CCD camera. Upon merging images for DAPI, anti-CD45-FITC, and anti-EpCAM-PE fluorescence, tumor cells which are positive for nuclei and positive for EpCAM are observed captured on the sides of the pore perpendicular to the applied field direction.

[0065] The experiment was repeated using anti-CK-PE instead of anti-EpCAM-PE. H1650 cells are observed to be magnetically captured along the pore edges perpendicular to the field direction. The cells are bright blue, due to the nuclear staining of DAPI, and are negative for CD45. The fluorescence intensity of anti-CK-PE, however, is faint. This is possibly due to low expression or the absence of the specific CKs targeted by the antibodies in the anti-CK-PE kit.

Experiment 3

Clinical Sample

[0066] The labeling discussed in the previous section was repeated on a clinical sample obtained from the Stanford Cancer Center. The blood sample (~10 mL) was drawn into an evacuated Cell Free DNA blood collection tubes with a proprietary cell fixative, from a cancer patient with Stage IV lung adenocarcinoma with widespread metastases in the brain and bone. 0.5 mL of the blood sample was processed in each experiment, using Magnetic Labeling Protocol 1, and the fluorescent staining sequence described above. In each case, captured cells were stained with DAPI and anti-CD45-FITC. Both anti-EpCAM-PE and anti-CK-PE were evaluated.

[0067] Merged images of tumor cells captured from the patient sample on the sifter surface and labeled with DAPI, anti-CD45-FITC, and anti-EpCAM-PE were inspected. The captured cells are bright blue due to the nuclear staining of DAPI and are located on the edges of the pore perpendicular to the applied field direction. Both anti-CD-45-FITC and anti-EpCAM-PE fluorescence is weak, indicating that white blood cells are not captured on the sifter surface, and that EpCAM expression is low compared to H1650 cells, or the fixation process interferes with anti-EpCAM binding to the cell surface.

[0068] When cells are stained with anti-CK-PE instead of anti-EpCAM-PE, merged fluorescent images reveal captured tumor cells that are positive for both nuclei and CK. The tumor cells are once again found on the edges of the pores perpendicular to the applied field direction. Since only one array could be imaged at a time and each merged image requires 3 image acquisitions of 1 second of exposure time with manual changing of the filter set in between each acquisition, only 30 pore arrays were imaged. 82 CTCs were counted. Since there are 544 pore arrays with four 50 μm pores each, assuming that the CTCs are evenly distributed throughout the pores, this yields a CTC count of 3000 CTCs/mL of blood. A microscope with automated paneling functions capable of rapidly imaging the sifter surface can expedite CTC enumeration from patient samples. Furthermore, in all experiments the background fluorescence is appreciable, especially for red fluorescence. The background fluorescence intensity is highest on the sifter surface, and lowest inside the pores where tumor cells are usually captured. This is attributed to the reflectance configuration of the microscope, in which excitation and image collection is done from the top. The background fluorescence, which makes tumor cells appear less bright, may be reduced significantly by an alternative microscope configuration in which the cells are excited by bottom illumination and imaged on top. This would result in cells captured only within the pores from being excited and imaged by the camera.